Origin and fine-tuning of effector CD8 T cell subpopulations in chronic infection.

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Abstract:

Persisting stimulation can skew CD8 T cells towards a hypofunctional state commonly referred to as T cell exhaustion. This functional attenuation likely constitutes a mechanism which evolved to balance T cell mediated viral control versus overwhelming immunopathology. Here, we highlight the recent progress in defining the genetic mechanisms and factors shaping the differentiation of exhausted CD8 T cells. We review how the transcription factor Tox imposes an exhausted phenotype in the Tcf1+ progenitors and how CD4 help fine-tunes the effector subsets that emerge from this progenitor population. Both processes critically shape the spectrum of effector function performed by CD8 T cells and the level of resulting virus control. Finally, we discuss how these insights can be exploited to boost the immune response in chronic infection and cancer.

Highlights:

- Tox is a lineage defining, T-cell exhaustion associated transcriptional factor.
- CD4 helper T cells support the maintenance of effector but not proliferation competent progenitor cells in chronic infection
- CD4 T cells fine-tune the effector subsets that emerge from the progenitor population
- IL-21 produced by CD4s supports the formation of the critical Cx3cr1+ effector cells.

Introduction:

Viruses like human immunodeficiency virus (HIV) or the Hepatitis B and C virus (HBV and HCV) as well as certain strains of Lymphocytic choriomeningitis virus in mice have the capacity to bypass protective immunity and establish chronic infections. This goes along with a tightly controlled partial stalling of the antigen-specific CD8 T-cell response known as the phenomenon of T-cell exhaustion [1]. The latter was first described in the 90s, when antigen-specific T cell responses in mice infected with persistent strains of LCMV (Lymphocytic choriomeningitis virus) were evaluated [2-4]. Later T cell exhaustion was observed in a variety of human persistent viral infections [5-10] and cancer [11-17]. The term was originally coined to highlight the circumstance of failed protection and vanishing anti-pathogen immunity. Nonetheless, we know now that a certain level of protective function is often retained over long periods and causes for instance the late onset of viral T-cell epitope escape variants occurring in HIV, HCV and Simian immunodeficiency virus (SIV) infections [18,19]. Similarly, SIV-infected rhesus macaques showed significant gains in virus titers following the removal of CD8 T cells [20-22]. This also underlines the presence of a functional T cell compartment. In contrast to the original loss of function concept, it becomes more and more accepted that T cell exhaustion merely reflects a differentiation process during which T cells adjust their effector capacity to the requirements and particular conditions found in persistent infection [23].

A key feature of exhausted T cells is that they mediate a critical level of long-term virus control, but cause less immunopathology than normal effector T cells [24,25]. This functional adaptation is achieved through the induction and epigenetic imprinting of an alternative transcriptional program. Exhausted CD8 T cells display decreased cytokine production (e.g. IL-2, IFNγ, TNFα) and increased co-expression of receptors negatively modulating their function – e.g. PD-1, TIM3, LAG3 and CTLA4, (**Figure 1**) [23,26-31]. High-antigen load and prolonged antigen exposure are the key factors that drive T cell into this specific functional

stage. This effect is further modulated by cytokines (e.g. IL-10 and TGF-β) and inhibitory receptor signalling [32,33].

Another critical feature of exhausted T-cell populations in chronic infections is that they are maintained over very long and often indefinite periods of time. The cells responsible for this long-term maintenance are proliferation competent Tcf1+ progenitor T cells [34-40]. These cells constantly generate exhausted effector cells, which are short-lived as it is the cause for effector T cells found in acute infection. Even though the Tcf1+ progenitors show some similarities with memory precursor T cells found in acute infections (e.g. both express Tcf1), the progenitors in chronic infection display typical signatures of T cell exhaustion including PD-1 expression [34]. Most importantly, this population is also very critical for immunotherapy, as PD-1 blockade stimulates the function of the progenitors resulting in increased numbers of antigen-specific effector cells [41,42].

A better understanding of the mechanisms that control CD8 T cell dynamics, differentiation, and protective potential is a prerequisite for making more effective immunotherapies against chronic viral infections and cancer. Significant progress has recently been made that include in depth characterizations of the progenitor population [42-46], the discovery of Tox as the first exhaustion specific transcriptional factor driving the epigenetic reprogramming of the progenitors in the early infection phase [47-52], an improved resolution of the diversity of exhausted effector T cell subpopulations [44,46], an advanced characterization of the role of helper T cells in fine-tuning the effector CD8 T cell compartment in chronic infection [44,46,53,54] and finally the demonstration of novel human memory CD8 T cell populations that express Tox and show a gene expression profile typically seen during T cell exhaustion [55,56]. These aspects will be reviewed in the subsequent sections.

Main text:

Transcriptional and epigenetic programming of antigen-specific T cells in chronic infection occurs primarily in the progenitor population.

While the phenotypic features of exhausted effector T cell populations are well established, a major challenge is to fully understand the processes that generate and maintain this phenotype. The majority of T cells found in exhausted T cell populations are terminally differentiated cells, which like their counterparts found in non-exhausted T cell populations have a short life-span and limited or no proliferative capacity. In contrast, the Tcf1+ progenitor population bears stem-like features and has the potential to form new effector T cells. Several years ago, we and others have observed that the proliferation competent progenitors carry signatures of exhaustion, which they stably transfer to their effector progeny [28,30,34,35,37,57]. This phenotype stability was owed to epigenetic imprinting and persisted even after resolution of the infection or check-point inhibition. This signalled that the progenitors are already committed to give rise to exhausted T cells but so far, we have only limited insights into how this commitment occurs.

Persisting T-cell receptor (TCR) signalling is a leading mechanism inducing T cell exhaustion [26,58]. Mechanistically, this is achieved via the induction of multiple TCR responsive transcriptional factors among which are NFAT, NR4A2, IRF4 and BATF [59-62]. However, the engagement of these factors is not specific to chronic infection as they also play a key role in acutely resolved infections. In contrast, several laboratories identified in parallel last year that the transcription regulator Tox (Thymus High Mobility Group Box Protein Tox) plays a highly specific role during T cell exhaustion in chronic mouse and human infection [47-51]. In contrast, though Tox can become transiently upregulated during acute infection in mice and human [48,63] and following strong ex-vivo stimulation, it was not reported to persist or affect the differentiation of bona-fide human or mouse effector T cells formed in acute infection [47-51]. In chronic infection, Tox attunes the transcriptional and epigenetic landscape of the

progenitor population. This affects genes such as Nr4a2, Pdcd1, Cd244, Lag3, ID3, and Havcr2 [47]. Thus, the Tox-induced epigenetic reprogramming of the progenitors explains the maintenance of the exhaustion signature following their re-activation through PD-1 blockade [64] or upon resolution of the infection [35].

Without Tox, the progenitors do not acquire the transcriptional signature of exhausted T cells and their progeny continues to display an acute phenotype. Initially this results in heightened virus control and massively augmented immunopathology [47]. The latter underscores the tissue protective role of T cell exhaustion. Despites these clearly beneficial for improved effector function aspects of removing Tox, a major limitation arises from the fact that the Tox-deficient progenitors fail to be maintained and are lost over time. As a consequence, the Tox-deficient pathogen-specific T cell populations decline sharply in the chronic phase of infection. Accordingly, the retention of an acute phenotype in the absence of Tox does not condition the progenitors to persist in an environment of chronic inflammation. Likely, the lack or reduced expression of inhibitory receptors on Tox-deficient progenitors, deprives those cells of mechanism counterbalancing their persistent stimulation. Thus, they are potentially driven into terminal differentiation. The complexity of Tox-mediated conditioning of CD8 T cell in chronic infection makes Tox a challenging candidate for direct therapeutic intervention. Nevertheless, Tox provides a promising starting point for the identification of downstream mechanisms regulating progenitor maintenance versus effector function. Importantly, Tox was reported to be expressed in human effector memory CD8 T cells specific for persistent viruses such as Cytomegalovirus (CMV), Epstein–Barr virus (EBV) and HIV, but not among influenza specific memory cells [65]. This further highlights the cross-species mode of action of Tox and the translational potential of its downstream regulatory networks.

CD4 T cell help is dispensable for maintaining functional progenitor T cells in chronic infection.

CD4 T cell help has long been known as a prerequisite for sustained antigen-specific CD8 T cell responses in chronic infection. In fact, it was recognized very early that the elimination of CD4 T cells from chronic LCMV infections in mice goes along with a much more prominently exhausted phenotype in antigen-specific CD8 T cell populations [3]. Therefore, the depletion of CD4 T cells is a common experimental approach to study T cell exhaustion. Nonetheless, it remained until recently unclear how CD4 T cells shape and impact the progenitor cells and their progeny. [66-70]. Based on observations in acute infection, where the presence of CD4 help is necessary for the formation of functional and long-lasting memory [71-73], a critical role of CD4 help for supporting the maintenance and function of the progenitors in chronic infection appeared to be a likely scenario. This would be well in line with the known numerical decline and deteriorating phenotype of persistently stimulated CD8 T cells in absence of CD4 help. In sharp contrast, we and other have shown recently that not the Tcf1-expressing progenitors but their terminally differentiated progeny is lost upon CD4 deprivation [44,53]. Surprisingly, side by side comparison between progenitors formed with or without of CD4 help showed identical transcriptional profiles [44]. Consistent with this, upon restoration of antigen-specific CD4 T cell help, the progenitors quickly repopulated the effector compartment with newly generated effector cells as indicated by their increased expression of Ki67. This indicated the general integrity of the progenitors in absence of CD4 help. What remains nonetheless unclear is if the progenitors fail to generate the differentiated effector progeny or if the survival of newly generated effector cells is impaired in absence of CD4 help. Very interestingly, this help independent survival applied only to progenitors with an exhausted phenotype but not those with a normal or polyfunctional one [44]. We had shown previously that such populations can be formed in chronic infection when antigen is presented in low quantities [33]. In such T cell populations both the progenitors and the terminally differentiated effector T cells lack the expression of inhibitory receptors and have augmented cytokine production capacity. Surprisingly, these progenitors vanish in the absence of CD4 help [44] as it was reported for conventional memory T cells [72]. This underlines functional differences between the two types of progenitor cells that goes far beyond the known phenotypic

differences. However, the exact genetic and functional differences among progenitors with or without an exhausted phenotype and of memory-precursor cells remain to be examined.

Helper T cells fine-tune the effector CD8 T cell subset distribution in chronic infection.

Defining the range and diversity of effector T cell states in chronic infection and the factors controlling their formation has long been of utmost importance. Previously, this was successfully analyzed at the level of the entire population [74], but the combination of single-cell RNA sequencing (scRNA-seq) with classical immunological techniques provides certain advantages. In contrast to bulk population sequencing, scRNA-seq has the ability to deconvolute cellular heterogeneity within mixed populations with still undefined subpopulation-specific markers [75]. In recent parallel studies by several laboratories, the application of scRNA-seq allowed the full grasp of the this phenotypic and functional diversity of CD8 T cells [44,53,54]. Upon clustering single-cell transcriptomes of P14 T cells recovered from chronically infected hosts with or without CD4 depletion, 5 discrete cell subpopulations were identified based on their gene expression profiles [44]. One of these populations represented the progenitors (expression of Tcf7, Slamf6), one population effector cells without an apparent signature of exhaustion (Cx3cr1, Gzmb, Tbx21) and three populations represented effector cells with varying degree of exhaustion (Pdcd1, Cd244 and Cd160). The lack of CD4 help shifted the percentage-wise representation of differentiated effector cells from Cx3cr1+ to populations with pronounced exhaustion signature [44,53,54]. Interestingly, this affected negatively not only the numbers of the Cx3cr1+ effector cells without apparent signs of exhaustion, but also of those with comparatively low degree of dysfunctional signature. Nevertheless, the absence of CD4 help did not impact the general capacity to form cells from all clusters found in control animals. While some precaution needs to be applied to the interpretation of cell dynamics on the basis of such a static shot analysis, the data nonetheless suggest that CD4 help shapes the composition of Tcf1-negative compartment and preferentially supports certain effector cell subsets. The Cx3cr1+ effector cells were reported

to be the recent progeny of the Tcf1 expressing progenitors, which could further continue their differentiation towards cells with dysfunctional phenotype [54]. Similar observations were made by Beltra and colleagues, who identified two distinct progenitor populations - one more tissue restricted and other more blood accessible [46]. The latter gradually lost Tcf1 as it divided and converted into effector cells with Cx3cr1+ effector signature. A study showing that diphtheria toxin receptor (DTR) induced ablation of the Cx3cr1-expressing population in chronic infection resulted in impaired viral control, highlights the significance of this population for the CD8 T cell response [53]. Additionally, PD-1 blockade increased the number of Cx3cr1+ effector CD8 T cells at least in the short-term [54]. IL-21 has long been known as one of the key mediators CD4 help in chronic infection, which absence causes severely impaired antigen-specific CD8 response and viral control [76-78]. It appeared that the formation or maintenance of the Cx3cr1+ effector cells in chronic infection depends on IL-21 produced by CD4 T cells [53]. The role in viral control of the remaining effector subpopulations apart from the Cx3cr1+ cells remains to be addressed. Additionally, better understanding of the regulation of these intra-populational transitions could provide a mean to promote some transitions (progenitors to Cx3cr1+ effector cells) while preventing others (Cx3cr1+ effector cells to cells with exhaustion signature).

The chance and challenge of developing subpopulation focused manipulation strategies.

The advances in our understanding of T cell differentiation and function gained in experimental model systems were readily reconciled in human patients including related to immunotherapies in different tumor entities. This started with the successful translation of PD-1 blockade [79] followed by numerous other examples of signaling mechanisms and pathways that are shared between chronic LCMV infection in mice and human chronic infections [57]. For instance, shortly after the discovery of the reservoir function of the Tcf1-expressing progenitors in persistent CD8 T cell responses, their frequency was associated with prolonged

duration and efficacy of the response to PD-1 blockade [41,42]. Nevertheless, it was also observed that PD-1 blockade only transiently boosts the production of functional effector cells which retain an exhausted phenotype [64]. All these examples underline the enormous richness of the LCMV model as a test and developmental resource for new therapeutic strategies. Alongside, the recently described diversity of exhausted T cell populations [44,46] raises a major new challenge to develop and adopt strategies for targeted manipulation of individual subpopulations. This includes strategies to selective enhance the function of the progenitor cells. Moreover, based on the new insights on effector compartment diversity mentioned above, the efficacy of PD-1 blockade or classical check-point inhibition could potentially be improved if combined with a secondary subpopulation-targeting treatment. The latter might be aimed at stimulating the maintenance or function of the Cx3cr1+ effector cells for instance. Interestingly, those cells uniquely express the receptor for IL18 (subunits Il18r1 and Il18rap), which is not expressed by any other cell subpopulation [44]. This goes along with a previous report suggesting Il18r1 is downregulated in the process of T cell exhaustion [80]. IL-18 is well known for its ability to limit activation-induced cell death, promote proliferation and interferon gamma (IFNγ) secretion in CD8 T cells [81,82]. Moreover, IL-18 supplementation in tumor models increased effector function and decreased exhaustion signature in CD8 T cells [83]. Thus, IL-18 supplementation seems a promising strategy to boost Cx3cr1+ effector cell function, which effectiveness alone or in combination with immune checkpoint blockade remains to be explored in models of chronic infection and cancer.

Conclusion:

The recent advances in our understanding of the factors and mechanism shaping CD8 T cell phenotype and function in chronic infection shed new light on the process underlying the phenomenon of T cell exhaustion (**Figure 2**). This includes the discovery of Tox as the first exhaustion specific transcriptional factor and the existence of a spectrum of effector cells with varying degree of exhaustion, including the critical Cx3cr1+ effector subpopulation. A key question which still remains unanswered is to what extent and up to what point T cell exhaustion can be reversed. The recent progress in genome-editing technologies allows for targeted manipulation of exhaustion specific genes. However, the simple deletion of exhaustion associated genes in particular in progenitor cells can reduce the overall survival and maintenance of the T cell population. This critical side effect was shown not only for Tox, but also for PD1 and IRF4 [47,48,61,84]. Theoretically, one would need to develop strategies where the progenitor cells are kept from undergoing terminal differentiation. As discussed above, this could involve the retention of signalling mechanisms such as PD-1 that supports the maintenance of the progenitors. At the same time, one would need on ensure that these exhaustion specific programs are not passed on to the effector cells. Such a combined strategy would preserve the progenitors while maximizing the effector capacity of their progeny.

Given the critical role of epigenetic mechanisms in the stable fixation of the exhausted phenotype, it will be of key importance to either identify pathways to overcome these mechanisms or to prevent their enforcement in first place. This was for instance shown for cells lacking Dnmt3a [85]. Additionally, approaches that directly promote the function of the less exhausted effector T cell subpopulations, like IL18 supplementation, can prove beneficial for improved CD8 T cell responses in viral infection and cancer.

Altogether, the field of T cell exhaustion and immunotherapy has reached an exciting stage. We have a detailed view of the origin and propagation of the exhausted phenotype. Moreover, we have identified key molecules and signaling pathways that drive this phenotype.

Nevertheless, we are still lacking approaches to effectively overcome or prevent T cell exhaustion in particular in clinically applicable fashion.

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Figure Legends

Figure 1. Schematic illustration of the time-dependent abundance of different CD8 T cell subpopulations in chronic infections.

Infections that induce T cell exhaustion typically generate diverse CD8 T-cell subpopulations. The early phase of infection often includes a larger fraction of cells with a polyfunctional phenotype (green). Then, over time cells with different level of functional impairment (yellow, orange, and red) become more prevalent. These subsets can be categorized into cells that have lost their ability to secrete IL-2 (yellow), as well as TNFα alone (orange) or in combination with IFNγ (red) secretion [70,86-88]. These changes go along with increased co-expression levels multiple inhibitory receptors (e.g. PD-1, TIGIT, Lag-3, Tim-3 and CTLA-4), which upon their engagement suppress T cell function in response to environmental stimuli [29,32,63,89-91]. How these changes occur at a cellular level remains still incompletely understood. It is widely believed that cells transition over time from a more functional towards more exhausted phenotype (cell conversion model). However, recent observations that non-exhausted T cell populations fail to be maintained in chronic infection [47] suggest that these changes may also results from a selective outgrowth or preferential survival of exhausted T cell populations over non-exhausted cells (selective survival model). Finally, a combination of 'cell conversion' and 'selective outgrowth' is also possible.

Figure 2. Schematic illustration of the impact of CD4 helper cells on CD8 T cell subpopulation diversity in chronic infection.

A and B show the CD8 T cell population composition with or without CD4 help respectively. Long-term maintenance of the population is secured by a proliferation competent Tcf1+ progenitor population [34-40]. The exhaustion specific transcriptional factor Tox drives transcriptional and epigenetic reprograming of the progenitors [47-51]. Their phenotype and

epigenetic profile are then passed onto their Tcf1- effector progeny. The effector compartment predominantly consists of cells with varying degree of dysfunctional signature (i.e. expression of PD1+, CD160+, CD244+), but also Cx3cr1+ cells with no apparent threats of T cell exhaustion [44,46,53,54]. These Cx3cr1+ effector cells are considered as the recent decedents of the progenitors [54]. The generation or maintenance of the Cx3cr1+ effector cells is highly CD4 help dependent, as their numbers dropped by more than 120-fold after CD4 depletion [44] . The CD4 help provided in this case occurs potentially through IL-21-dependent mechanism [53]. The absence of CD4 help also negatively impacts the numbers of effector cells with less pronounced signature of T cell exhaustion [44]. The unique expression of IL-18R on the Cx3cr1+ effector population, could potentially be exploited to provide stimulation signals to these cells [44].

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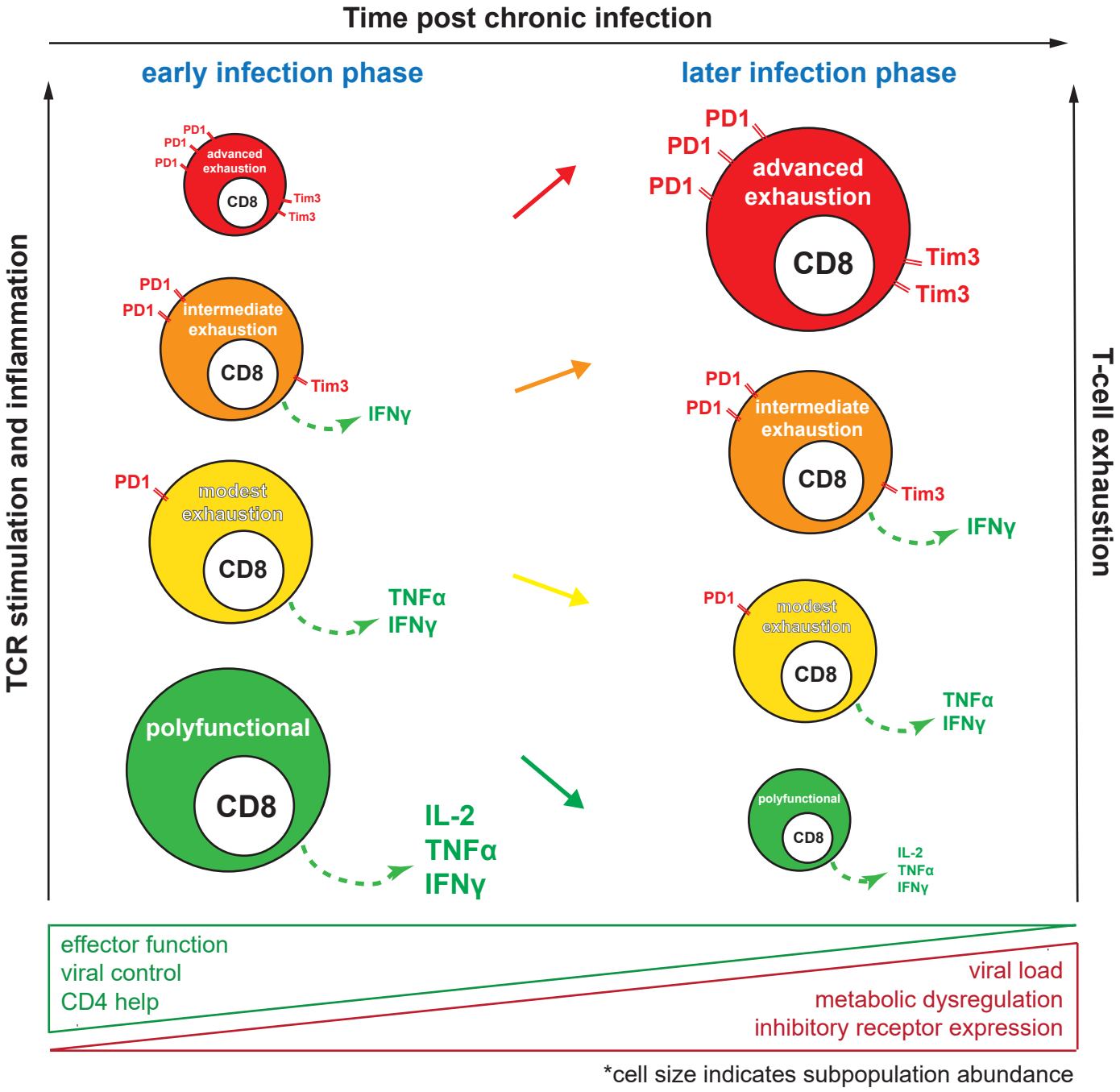
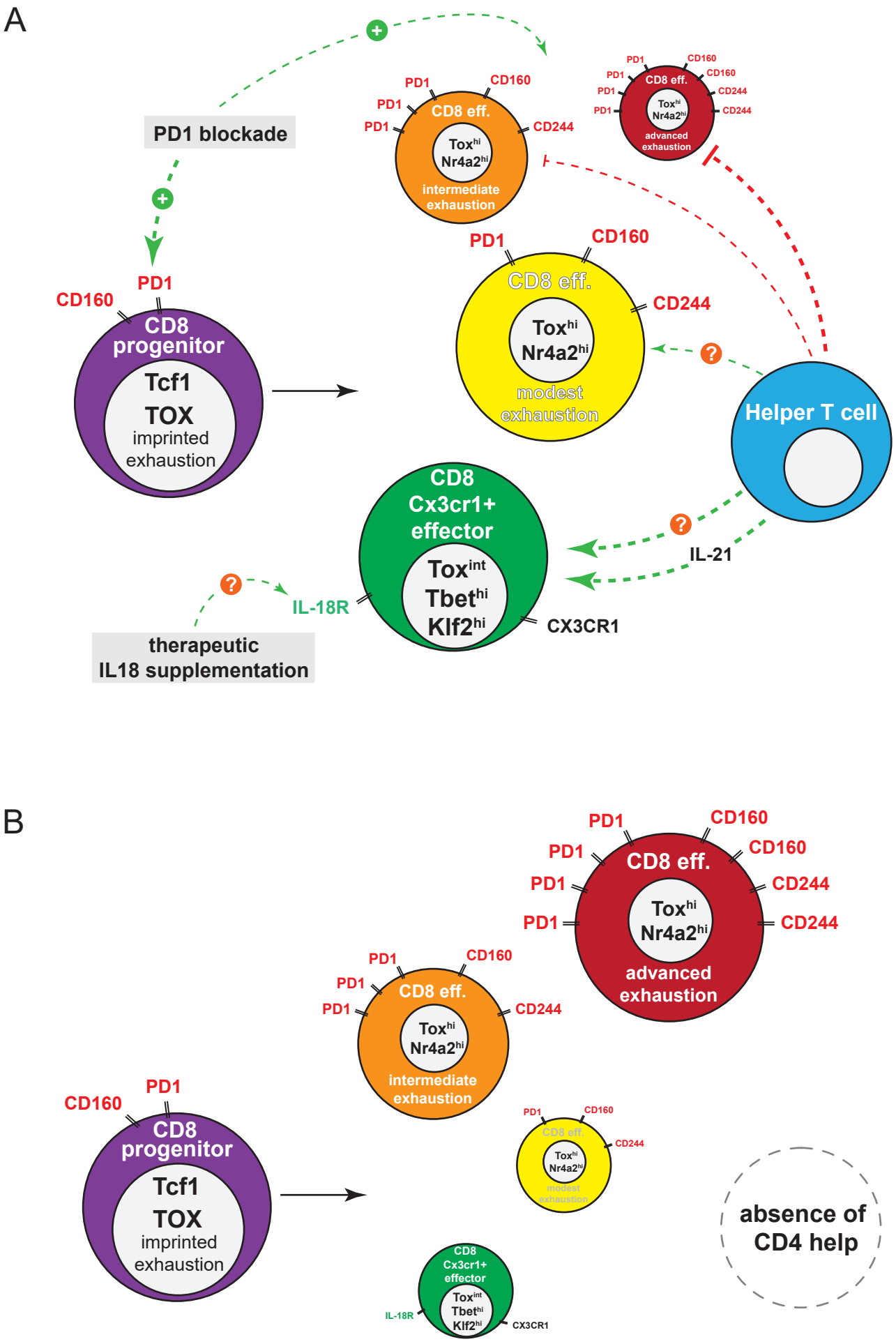
Figure 1



*cell size indicates subpopulation abundance

Figure 2


A
PD1 blockade
PD1
CD160
CD8 progenitor
Tcf1
TOX
imprinted
exhaustion
PD1
CD160
CD8 eff.
CD244
Tox^hi
Nr4a2^hi
intermediate
exhaustion
PD1
CD160
CD8 eff.
CD244
Tox^hi
Nr4a2^hi
modest
exhaustion
PD1
CD160
CD8 eff.
CD244
Tox^hi
Nr4a2^hi
advanced
exhaustion
Helper T cell
IL-21
CD8
Cx3cr1+
effector
Tox^int
Tbet^hi
Klf2^hi
CX3CR1
IL-18R
therapeutic
IL18 supplementation
B
PD1
CD160
CD8 progenitor
Tcf1
TOX
imprinted
exhaustion
PD1
CD160
CD8 eff.
CD244
Tox^hi
Nr4a2^hi
intermediate
exhaustion
PD1
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CD8 eff.
CD244
Tox^hi
Nr4a2^hi
advanced
exhaustion
CD8
Cx3cr1+
effector
Tox^int
Tbet^hi
Klf2^hi
IL-18R
CX3CR1
absence of
CD4 help
*cell size indicates subpopulation abundance